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Complete List of Authors:	Berton, Paula; National Council for Scientific and Technical Research (CONICET), Domínguez-Romero, Juan C.; University of Jaén, Analytical Chemistry Research Group WUILLOUD, RODOLFO; National Council for Scientific and Technical Research (CONICET), LISAMEN Sánchez-Calvo, Beatriz; Universidad de Jaén, Departamento de Bioquímica y Biología Molecular Carreras, Alfonso; Universidad de Jaén, Departamento de Bioquímica y Biología Molecular Barroso, Juan B.; Universidad de Jaén, Departamento de Bioquímica y Biología Molecular Valderrama, Raquel; Universidad de Jaén, Departamento de Bioquímica y Biología Molecular Gilbert-López, Bienvenida; University of Jaén, Analytical Chemistry Research Group; University of Jaén, Department of Physical and Analytical Chemistry García-Reyes, Juan F.; University of Jaén, Department of Physical and Analytical Chemistry Molina Diaz, Antonio; University of Jaén, Department of Physical and Analytical Chemistry
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Determination of nitrotyrosine in *Arabidopsis thaliana* cell cultures with a mixed-mode solid-phase extraction cleanup followed by liquid chromatography time-of-flight mass spectrometry

Paula Berton[a,b,c], Juan C. Domínguez-Romero[a], Rodolfo G. Wuilloud[b,c], Beatriz Sánchez-Calvo[d], Mounira Chaki[d], Alfonso Carreras[d], Raquel Valderrama[d], Juan C. Begara-Morales[d], Francisco J. Corpas[d], Juan B. Barroso[d], Bienvenida Gilbert-López[a], Juan F. García-Reyes[a] and Antonio Molina-Díaz[a,*]

[a]*Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, Campus Las Lagunillas, Edif. B-3, 23071 Jaén, Spain.*
[b]*Analytical Chemistry Research and Development Group (QUIANID), Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Padre Jorge Contreras 1300, Parque Gral. San Martín, C.P. M5502JMA Mendoza, Argentina.*
[c]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.*
[d]*Grupo de Señalización Molecular y Sistemas Antioxidantes en Plantas, Unidad asociada al CSIC (EEZ), Departamento de Bioquímica y Biología Molecular, Universidad de Jaén, Spain.*

Keywords: nitrotyrosine, *Arabidopsis thaliana*, nitrosative stress, liquid chromatography, mass spectrometry, solid-phase extraction.

*Corresponding author: Prof. Antonio Molina-Díaz. Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, 23071 Jaén, Spain. Tel.: (+34) 953 212147; Fax: (+34) 953 212940. E-mail:amolina@ujaen.es

Abstract

In this work, a method for the determination of trace nitrotyrosine (NO_2Tyr) and tyrosine (Tyr) in *Arabidopsis thaliana* cell cultures is proposed. Due to the complexity of the resulting extracts after protein precipitation and enzymatic digestion and the strong electrospray signal suppression displayed in the detection of both Tyr and NO_2Tyr from raw *Arabidopsis thaliana* cell culture extracts, a straightforward sample cleanup step was proposed. It was based on the use of mixed-mode solid-phase extraction (SPE) using MCX-type cartridges (Strata™-X-C), prior to identification and quantitation using fast liquid chromatography electrospray time-of-flight mass spectrometry (LC-TOFMS). Unambiguous confirmation of both aminoacids was accomplished with accurate mass measurements (with errors lower than 2 ppm) of each protonated molecule along with a characteristic fragment ion for each species. Recovery studies were accomplished to evaluate the performance of the SPE sample preparation step obtaining average recoveries in the range 92–101%. Limit of quantitation (LOQ) obtained for NO_2Tyr in *Arabidopsis thaliana* extracts was 3 nmol L^{-1}. Finally, the proposed method was applied to evaluate stress conditions of the plant upon different concentrations of peroxynitrite, a protein-nitrating compound, which induces the nitration of Tyr at the nanomolar range. Detection and confirmation of the compounds demonstrated the usefulness of the proposed approach.

Keywords: nitrotyrosine, *Arabidopsis thaliana*, nitrosative stress, liquid chromatography, mass spectrometry, solid-phase extraction.

1. Introduction

Tyrosine (Tyr) nitration is becoming increasingly recognized as a prevalent, functionally significant post-translational protein modification (PTM), which can occur in cells during oxidative stress and over-production of nitric oxide [1]. This modification is involved in the control of fundamental cellular processes including cell cycle, cell adhesion and cell survival, as well as cell proliferation and differentiation [2]. The addition of NO_2 group to the ortho-position of Tyr confers particular physicochemical properties to the modified amino acid and the corresponding proteins, as a consequence of pK_a reduction of about three units [3]. These changes in protein conformation may have important functional consequences, such as a loss, an increase, or no effect on protein function [4-7]. Elevated levels of 3-nitrotyrosine (NO_2Tyr) have been reported in a range of pathological conditions including inflammatory, neurodegenerative, and cardiovascular disorders, among others [8,9]. Moreover, emerging data indicate a novel biological function for Tyr nitration in the regulation of immune responses [1]. Therefore, in mammals Tyr nitration is being intensively studied because it can be used as a biomarker not only of nitrosative stress, but also of certain pathological and physiological conditions [10,11]. Additionally, new studies emphasize the possible involvement of Tyr nitration in signaling pathways mediated by NO [1].

On the other hand, in plants the information available on protein nitration under normal conditions is rather limited [12]. Even though previous data indicate the existence of a basal nitration present in the plant tissues analyzed, there are published data which indicate that an increase in the number of proteins or an intensification of specific proteins resulting from Tyr nitration could be considered as an indicator of nitrosative stress in plants [13,7,14,15]. Therefore, protein Tyr nitration might be a good

starting point in the search of nitrosative stress markers in plant cells [13]. Nevertheless, since the actual number of nitrated Tyr residues in proteins is unknown, it is by far more preferable to use molar ratio of nitrated Tyr residues to non-nitrated Tyr residues [16]. However, the overall concentration of nitrated Tyr residues is typically low [9]. Hence, assays applied to the analysis of NO_2Tyr in biological samples must offer a low limit of detection, accuracy and precision.

Detection of NO_2Tyr in biological samples has been extensively reported in the literature. These methods fall into two basic categories: molecular analysis using NO_2Tyr antibody-staining techniques [13] and chemical analysis using HPLC and GC [14], mainly using mass spectrometers as detectors. The source and nature of analytical problems, shortcomings and pitfalls associated with NO_2Tyr determination have been reviewed by Duncan [17] and Tsikas [16,18]. The main drawbacks are both the low abundance of nitrated species and lack of efficient enrichment methods [2].

Mass spectrometry (MS) is a powerful analytical technique with inherent selectivity, sensitivity and precision when applied to NO_2Tyr determination. Moreover, NO_2Tyr immunoassays, unlike GC-MS and LC-MS based-methods, cannot provide important information about NO_2Tyr/Tyr ratio [17,19]. In view of the complexity inherent in the determination of NO_2Tyr, and the confounding results evident in the literature, MS has thus been adopted by several groups [18]. Furthermore, comparing with GC-based methods, LC-MS methods offer advantages such as that it is no longer necessary to modify the analyte to impart volatility. Because chemical manipulation can be eliminated, sample handling, the potential for side-reactions, losses and contamination are also minimized [17]. These complex matrices require, however, a careful consideration in order to evaluate and eliminate matrix effects when developing an LC-MS assay, particularly because of matrix effects/signal suppression, the Achilles'

heel of quantitative LC–Electrospray(ESI)-MS [20]. In LC-ESI-MS, methods skipping sample cleanup stages lead to poor analytical performance, in particular, when complex matrices are addressed and sensitive methods are needed. In the present work, a sensitive, simple and specific sample preparation method based on mixed-mode solid-phase extraction (SPE) was developed for the accurate quantification of trace NO_2Tyr in plant tissues by LC-TOFMS using *Arabidopsis thaliana*, as model sample.

2. Materials and methods

2.1. Reagents and Materials

Tyrosine (Aldrich) and 3-nitro-L-tyrosine (Aldrich) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the studied compounds (1.77 mmol L^{-1} of nitrotyrosine and 1.10 mmol L^{-1} of tyrosine) were prepared in water and stored at −20 ºC. HPLC-grade solvents acetonitrile (Chromasolv® Gradient) and methanol (Chromasolv® for HPLC) were purchased from Sigma-Aldrich. Formic acid was obtained from Fluka (Buchs, Switzerland). A solution of 5% (v/v) ammonium hydroxide (Sigma-Aldrich) in methanol was employed in SPE procedure. A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water. The SPE cartridges evaluated for comparing cleanup were Strata™-X-C cartridges with a capacity of 30 mg, (Phenomenex, Torrance, CA, USA); AccuBOND^{II} SCX cartridges (200 mg, 3 mL) were acquired from Agilent Technologies (Waldbronn, Germany); Oasis MCX SPE cartridges (150 mg, 6 mL) and Oasis HLB (200 mg, 6 mL) were purchased from Waters (Milford, MA, USA). Additionally, a Supelco (Bellefonte, PA, USA) Visiprep™ SPE vacuum system was also employed.

2.2. Sample preparation and treatment

Arabidopsis thaliana L. (Columbia ecotype) cell suspension culture was kindly provided by the Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Salamanca (Spain). The culture was maintained in 200 mL of liquid growth medium [21,22] by gentle agitation at 120 rpm and 24 ºC under continuous illumination (50 μE m^{-2} s^{-1}) in an incubator shaker. Cells were sub-cultured with a one-twentieth dilution every seven days. The treatment of the cell culture was performed as described by Chaki *et al.* [23,24]. The cell culture was treated with different concentrations of peroxynitrite by infusion for one hour in the same cell culture conditions. After an hour, cell suspension culture was grounded and homogenized in liquid nitrogen using a mortar and pestle. The resulting powder was suspended into 1/2 (w/v) digestion buffer (50 mmol L^{-1} sodium acetate, pH 6.5) according to Hensley *et al.* [25]. Homogenates were then filtered through one layer of Miracloth (Calbiochem, San Diego, CA, USA) and centrifuged at 3000 g for 10 min. The supernatant proteins were then precipitated by the addition of 10% trichloroacetic acid (TCA). After incubation at 4 ºC for 20 min, the samples were centrifuged at 14,000 g for 10 min. Protein pellets were washed twice with acetone at –20 ºC, air-dried, and re-suspended in 1 mL of digestion buffer containing 4 mg of pronase (Calbiochem), and incubated at 50 ºC for 30 h with gentle stirring. The digested samples were treated with 10% TCA at 4 ºC for 20 min followed by centrifugation at 14000 g for 10 min. The pH of the supernatant was adjusted to 3. The supernatants were passed through 0.45 µm PVDF filter.

2.3. Mixed-mode solid-phase extraction cleanup

Strata™-X-C cartridges cation-exchange cartridges with a capacity of 30 mg, with a mixed-mode stationary phase (strong cation-exchange and reverse-phase) were used to perform the SPE-based cleanup. The cartridges were placed on a vacuum SPE manifold being preconditioned with 1 mL of methanol and 1 mL of 0.1 N HCl in water

at a flow rate of 2 mL min^{-1}. Subsequently, 2.5 mL of plant extract (previous adjustment to pH 3) was loaded onto the SPE cartridge, at a flow rate of 1 mL min^{-1}. Finally, the sample was eluted into the test tube using twice 2 mL of 5% (v/v) ammonium hydroxide in methanol at 1 mL min^{-1}. The eluate pH was then neutralized by vacuum evaporation of the ammonium hydroxide. Samples were evaporated until near dryness by a gentle nitrogen stream and reconstituted with 500 µL of methanol:H_20 (20%, v/v) (final preconcentration factor 5:1) prior to analysis. The extract was finally filtered through a 0.45 µm PTFE filter (Millex FG, Millipore, Millford, MA, USA). For validation and quantitation purposes, matrix-matched standards were prepared by spiking the extracts with appropriate volume of NO_2Tyr working standard solution before the SPE extraction procedure.

Additional experiments were also performed using cation-exchange and reverse-phase type SPE cartridges). Two cation-exchange cartridges (AccuBOND^{II} SCX (200 mg, 3 mL) and Oasis MCX SPE cartridges (150 mg, 6 mL)) were also tested although they were not selected as the final optimized method. The cation-exchange SPE cartridges were washed with MeOH (5 ml) and 5 mL of 0.1 M HCl in water at a flow rate of 2 mL min^{-1}. Subsequently, 10 mL of plant extract (previous adjustment to pH 3) was loaded onto the SPE cartridge, at a flow rate of 1 mL min^{-1}. Finally, the sample was eluted into the test tube using twice 2.5 mL of 5% (v/v) ammonium hydroxide in methanol at 1 mL min^{-1}. The resulting extract were evaporated until near dryness by a gentle nitrogen stream and reconstituted with 2 mL of methanol:H_20 (20%, v/v) prior to LC-MS analysis.

Besides, a hydrophilic-lipophilic balanced Oasis HLB cartridge was also tested (200 mg, 6 mL). The cartridge was washed with MeOH (5 ml) and 5 mL of mQ water at a

flow rate of 2 mL min^{-1}. Subsequently, 10 mL of plant extract (previous adjustment to pH 3) was loaded onto the SPE cartridge, at a flow rate of 1 mL min^{-1}. Finally, the sample was eluted into the test tube using twice 5 mL methanol at 1 mL min^{-1}. The resulting extract was evaporated until near dryness by a gentle nitrogen stream and reconstituted with 2 mL of methanol:H_20 (20%, v/v) prior to analysis.

2.4. Liquid chromatography electrospray time-of-flight mass spectrometry

The separation of the species from the whole SPE extracts was carried out using an HPLC system (consisting of vacuum degasser, auto-sampler and a binary pump) (Agilent 1290 Infinity, Agilent Technologies, Santa Clara, CA, USA). Optimization studies were carried out with standard mixtures performing chromatographic separation on an Agilent ZORBAX Eclipse XDB-C_{18}, Rapid Resolution HT (4.6 × 100 mm, 1.8 µm). For the elution, 0.1% (v/v) formic acid in high purity water (mobile phase A) and acetonitrile (mobile phase B) were used as solvents at a flow rate of 500 µL min^{-1}. The gradient program started at 5% B and after 2 min of isocratic run solvent B was increased linearly and reached 50% at 10 min, then 100% at 13 min. Finally, 100% B was kept constant for two minutes (until 15 min) and after the acquisition 10 min post time was set for the equilibration of the initial solvent composition. The column temperature was maintained at 24 ºC and an injection volume of 20 µL was used in all experiments.

The HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in positive or negative ion mode, using the following operation parameters: capillary voltage, ±4000 V; nebulizer pressure, 40 psig; drying gas flow

rate, 9 L $min^{-1;}$ gas temperature, 325 ºC; skimmer voltage, 65 V; fragmentor voltage (in-source CID fragmentation), 170 V in positive ion mode. LC−MS accurate mass spectra were recorded across the range of 50−1000 m/z. Accurate mass measurements of each peak from the total ion chromatograms (TICs) were obtained using an automated calibrant delivery system to provide the correction of the masses. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low flow (approximately 10 µL min^{-1}) of a calibrating solution which contains the internal reference masses purine ($C_5H_4N_4$ at m/z 121.050873) and HP-0921 ([hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at m/z 922.009798)). The full-scan data recorded was processed with Agilent Mass Hunter software (version B.04.00). Extracted ion chromatograms were obtained throughout the study using ±5 mDa mass window.

3. Results and discussion

3.1. Identification and confirmation of tyrosine and nitrotyrosine by LC–ESI-TOFMS: in-source CID fragmentation and accurate mass measurements

The fragmentor voltage is the parameter that establishes the extent in which in-source CID fragmentation is carried out, which may have a strong influence on the sensitivity and relative abundance of protonated molecules [26]. Due to the low masses of both Tyr and NO_2Tyr, the fragmentor voltage was set at 170 V (mild conditions), as a compromise value between sensitivity for quantitation and additional mass spectrum information for confirmation purposes. Using the selected conditions, useful fragmentation was obtained. Table 1 shows the fragmentation of Tyr and NO_2Tyr and the relative abundances of the different species formed.

Primary identification of both compounds was performed basically by retention time matching and accurate mass measurements of the targeted protonated molecules and their main fragment ions. By using high resolution mass spectrometry data with high mass accuracies, as those shown in Table 1, unambiguous identification of the targeted species was accomplished. For identification and quantitation purposes, extracted ion chromatograms (ECIs) were employed, using a mass-window width of 5 mDa ($[M+H]^+$ ± 5 mDa). The protonated molecule ($[M+H]^+$) was used for both identification and quantitation purposes for NO_2Tyr and Tyr. Accurate-mass data from additional fragment ions available for NO_2Tyr and Tyr were used for further confirmation. Figure 1 show LC-TOFMS mass spectra of Tyr and NO_2Tyr obtained in the positive ionization mode.

<Figure 1 and Table 1>

3.2. Sample treatment and recovery studies

After unsuccessful attempt of direct injection of the *Arabidopsis thaliana* extract (Figures 2 and 3), in order to eliminate additional interfering species from the sample extract, a SPE cleanup step was evaluated and included in the method. Although slightly more time-consuming, the improvement in chromatographic performance provided by the SPE step was significant. Additionally, the extraction method could be easily automated using a SPE–LC–TOFMS assembly, thus increasing the throughput and automation degree of the procedure. Inspection of the structure, solubility data, and acid/base properties of Tyr and NO_2Tyr suggests that it can be extracted by different mechanisms. For example, ionic interactions could be increased through pH variation. Furthermore, the aromatic side chain of Tyr and NO_2Tyr (Fig. 1) can be involved in stacking (non-polar) interactions with other aromatic side-chains; and the reactive hydroxyl group can be involved in polar interactions such as hydrogen bonding.

Therefore, different sorbent materials with non-polar, polar, or ion-exchange properties were evaluated.

Among strong cation-exchangers (SCX), a cartridge based on silica (AccuBOND^{II} SCX) was tested. It is generally employed to extract positively charged basic compounds. Moreover, this benzene-sulphonic acid-based sorbent has significant non-polar secondary interactions. Different cartridges with a mixed-mode stationary phase (MCX) with reverse-phase and cation-exchange dual functionality, such as Oasis MCX and Strata™-X-C were also evaluated. Besides, HLB cartridges, which have both hydrophilic and lipophilic properties, generally employed to extract a variety of polar and non-polar compounds, were also considered. The cleanest chromatograms were obtained when cation exchange-based materials were employed. Among these materials, best recoveries were obtained when Strata™-X-C cartridges were employed (91 and 83% recovery for Tyr and NO_2Tyr for Strata versus 61 and 46% for Tyr and NO_2Tyr with Oasis MCX), and the extracts obtained were particularly clean. Therefore, in order to maximize the retentive differences between the analytes and the vegetable matrix, Strata™-X-C cartridges were employed for isolate the analytes from the matrix. For the SPE step, 2.5 mL of vegetable matrix sample were selected as the loaded volume. The preconcentration factor achieved in the final extract (500 µL) was 5:1.

The pH is a significant variable when developing a SPE method. Interactions between the matrix components and the target analytes in biological samples may be disrupted by a change in pH [27]. Thus, spiked matrix stabilized at neutral (pH 7) and acidic (pH 3) pHs were evaluated for both MCX cartridges. A significant improvement on analytes recoveries was observed when Strata cartridges at acidic pH (85-90% recovery for Tyr and NO_2Tyr) were employed, comparing with those at neutral pH

(lower than 10% for both analytes). Therefore, pH from samples was adjusted to 3 before SPE.

Figure 2 shows a comparison of Total Ion Current Chromatograms (TICs) obtained from the raw *Arabidopsis thaliana* extract without further treatment (Figure 2a) and with the proposed SPE-based cleanup method (Figure 2b). The TICs accounts for the sum of signals for all coeluting ions at each individual acquired spectrum. This can be used as an indicator of the complexity of a matrix and to evaluate the degree of efficiency of a cleanup step. Note that the TIC obtained with the SPE procedure is cleaner (lower average signal) than the raw extract, even considering the 5:1 preconcentration factor, as shown in the chromatogram where major matrix peaks are baseline-separated and the TIC current is similar to the raw extract despite the preconcentration factor (5:1). With this SPE approach, the chromatogram region where NO_2Tyr is detected is free of several coeluting interfering species. In Figure 3, EICs for the detection of NO_2Tyr in the studied plant extracts obtained from (a) the raw extract without further treatment (500 nmol L^{-1} NO_2Tyr) and (b) with the proposed SPE-based cleanup method (100 nmol L^{-1} NO_2Tyr in the original *Arabidopsis thaliana* extract), are shown. It can be seen that at the studied concentration level (500 nmol L^{-1}), NO_2Tyr could not be detected in the raw extract due to strong signal suppression due to matrix coeluting components. In contrast, the identification of NO_2Tyr with the SPE approach was straightforward.

<Figure 2 and 3>

Even though after the sample treatment protocol a cleaner extract is obtained, the impact of the matrix on the ionization suppression/enhancement on the analytes was still significant. Therefore, a calibration with matrix-matched standards was employed throughout the study to minimize errors due to matrix effects.

<Table 2>

3.3. Analytical performance: in vitro nitration of Arabidopsis Thaliana cells

To evaluate the analytical features of the proposed method, calibration curves were constructed at different concentrations, in the range 10-500 and 50-2500 nmol L^{-1} of Tyr and NO_2Tyr respectively, using vegetable extracts to prepare matrix-matched standards at several concentration levels (2-100 and 10-500 nmol L^{-1} of Tyr and NO_2Tyr respectively), considering the SPE preconcentration factor. The results obtained are shown in Table 2 where the calibration curves are summarized together with the limits of quantitation (LOQs), matrix effects and relative standard deviation (RSD, %). The linearity of the analytical response across the studied range was excellent, taking into account that the calibration curves of the analyzed compounds showed correlation coefficients higher than 0.996. The RSD (n = 6) values for run-to-run study were 2.7 and 3.4% for Tyr and NO_2Tyr respectively. These results demonstrate the precision of the developed method and the potential of the proposed approach for quantitative purposes. The LOQs were estimated as the minimum concentration of analyte corresponding to a signal-to-noise ratio (S/N) = 10:1. This was experimentally calculated from the injection of matrix-matched standard solutions at low concentration levels, using the more abundant ion for each compound based on the signal from high-resolution EICs with narrow mass windows (targeted mass ± 5 mDa). The LOQ obtained for NO_2Tyr was 3 nmol L^{-1}. Compared to the concentration levels that were achieved in previous reported methods for other biological matrices and considering the complexity of the studied extract, the LOQs reported here can be considered very satisfactory for the targeted application [28]. In the case of Tyr, LOQ could not be calculated because it is already present at large excess compared to NO_2Tyr in the

studied samples. The LOQ of neat Tyr solvent standard was 10 nmol L^{-1} (without preconcentration step).

To evaluate the effectiveness of the extraction method, a recovery study was carried out. *Arabidopsis thaliana L.* cell culture was incubated with two different concentrations of pure peroxynitrite (1 and 5 mmol L^{-1}), which had been shown to mediate Tyr nitration [24]. After sample preparation (explained on section 2.2), the aliquots were spiked at different concentration levels (0.5 - 1 µmol L^{-1}) with the working standard solutions of Tyr and NO_2Tyr. The spiked samples were extracted with the SPE method described and then analyzed with the developed LC-TOFMS method. Due to the high concentration level differences between Tyr and NO_2Tyr (*ca.* 3 orders of magnitude) it was extremely difficult to accurately measure both Tyr and NO_2Tyr in the same run. This limitation is set by mass spectrometer, which usually features 2.5-3 orders of linear dynamic range. For this reason, and also to skip matrix effects for accurate Tyr quantitation, a 1:100 dilution of the extract was also analyzed which enabled the determination of Tyr without matrix effects, just by using external solvent-based calibration. A LC-ESI(+)TOFMS identification of NO_2Tyr in cell cultures exposed to peroxynitrite is shown in Fig. 4. The obtained recoveries rates for NO_2Tyr were in the range 92-101%, as shown in Table 3. These results show the feasibility of the studied extraction method for NO_2Tyr determination in the studied vegetable extracts. Besides, in the samples tested, both Tyr and NO_2Tyr were calculated for both experiments (1 and 5 mmol L^{-1} of peroxynitrite). Interestingly, a linear correlation tendency between concentration of peroxynitrite and NO_2Tyr/Tyr ratio was observed. This proportional increase in the concentration of NO_2Tyr when increasing the concentrations of peroxynitrite corroborates the use of NO_2Tyr as a marker of nitrosative stress in plants.

<Figure 4 and Table 3>

4. Conclusions

The present work described a new method based on SPE and LC-TOFMS for quantitative analyses of NO_2Tyr and Tyr in *Arabidopsis thaliana* cell culture extracts. The method is simple, since it involves a quick cleanup step with SPE employing polymer-based cartridges before measurement with LC-TOFMS. Satisfactory recoveries were obtained for both studied compounds. Moreover, the high sensitivity obtained with the proposed method compares well with previous LC-MS/MS methods described for the analyses of NO_2Tyr and Tyr in biological matrices. The potential of the proposed method was demonstrated by analyzing real samples with excellent selectivity and sensitivity, thus enabling the unambiguous identification, by means of accurate mass analysis, and quantitation of low levels of NO_2Tyr in *Arabidopsis thaliana* cell culture extracts. The proposed LC-TOFMS method also offers the possibility of performing a posteriori (non-target) analysis of the samples, such as the search and identification of others PTM-tyrosine compounds (such as sulfation, phosphorylation or carbonylation), involved in the regulation of a wide range of biological processes [29]. All the data are saved and can be re-examined to check for compounds that previously were not expected or were not subjected to control. This is an additional attractive feature that highlights the potential application of this method based on LC-TOFMS for studies related to PTM in biochemical laboratories worldwide.

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Table 1. Identification of tyrosine and nitrotyrosine by LC-TOFMS. Accurate mass measurements of the protonated molecules and the main fragment ions using *Arabidopsis thaliana* extracts[a]

Compound	t_R (min)	ion	Elemental compositions	Relative Abundance (%)	*m/z* theoretical	*m/z* experimental	Error	
							mDa	ppm
Tyrosine	3.43	$[M + H]^+$	$C_9H_{12}NO_3^+$	100	182.0817	182.0812	- 0.50	- 2.75
		$[M + H – NH_3]^+$	$C_9H_9O_3^+$	54	165.0546	165.0548	0.20	1.21
		$[M + H - HCOOH]^+$	$C_8H_{10}NO^+$	59	136.0757	136.0749	- 0.80	- 5.88
3-Nitrotyrosine [a]	7.99	$[M + H]^+$	$C_9H_{11}N_2O_5^+$	100	227.0662	227.0666	0.40	1.76
		$[M + H - HCOOH]^+$	$C_8H_9N_2O_3^+$	36	181.0608	181.0609	0.10	0.55

[a]Spiking level: 100 nmol L^{-1}.

Table 2. Analytical parameters for the detection of nitrotyrosine in *Arabidopsis thaliana* cell culture extracts by LC–TOFMS.

Compound	**Conc. range tested** (nmol L^{-1})	**Regression equation**	**Matrix effect**[a] (Δ %)	**Linearity** (r)	**LOQ** (nmol L^{-1})	**RSD** (%)[c]
Tyrosine	10 - 500	$y = 2.624 \times 10^3 C + 7.97 \times 10^3$	Not calculated[b]	0.9997	Not calculated[b]	2.7
3-Nitrotyrosine	50 - 2500	$y = 1.36 \times 10^3 C + 3.21 \times 10^4$	0.07 (-93)	0.9972	3	3.4

[a]Ratio: matrix-matched calibration slope/solvent calibration slope.
[b]Matrix effect and limits of detection for Tyr could not be calculated because *Arabidopsis thaliana* cell culture extracts already contained Tyr between 100-250 µmol L^{-1}. A 200:1 dilution was applied for quantitation purposes. The LOQ of Tyr in solvent standard using the proposed method is 10 nmol L^{-1}.
[c]Concentration level: 100 nmol L^{-1}. n = 6

Table 3. Recovery studies on *Arabidopsis thaliana* cell culture extracts treated with peroxynitrite (1 and 5 mmol L^{-1}).

Sample treatment	Nitrotyrosine				Tyrosine (µmol L^{-1})	NO_2Tyr/Tyr ratio[a]
	Spiking level (µmol L^{-1})	Found (µmol L^{-1})[a]	Recovery (%)[a]	RSD (%)[a]		
Treatment 1:	0	0.196	---	---	189.33	0.001035
1 mmol L^{-1}	0.5	0.673	96.7	6.3		
peroxynitrite	1	1.100	92.0	7.8		
Treatment 2:	0	0.580	---	---	117.78	0.00492
5 mmol L^{-1}	0.5	1.090	101.0	3.1		
peroxynitrite	1	1.551	98.2	5.2		

[a] n=3

Figure captions

Figure 1. LC-Electrospray TOFMS mass spectra of Tyr and NO_2Tyr acquired in the positive ionization mode.

Figure 2. Total ion chromatograms (TICs) from the plant extracts obtained from the raw extract (a) without further treatment and (b) with the proposed SPE-based cleanup method.

Figure 3. Extracted ion chromatograms (EICs) for the detection of NO_2Tyr in the studied *Arabidopsis thaliana* cell culture extracts: (a) EIC obtained from the raw extract without further treatment ([NO_2Tyr] = 500 nmol L^{-1}); and (b) EIC obtained with the proposed SPE-based cleanup method ([NO_2Tyr] = 100 nmol L^{-1}).

Figure 4. LC-ESI(+)TOFMS identification of NO_2Tyr in *Arabidopsis Thaliana* cell cultures exposed to peroxynitrite (nitrating compound). Left: extracted ion chromatogram (m/z 227.0667); right: Electrospray TOFMS spectrum including ions at *m/z* 227.0666 and *m/z* 181.0611 that provides the unambiguous identification of NO_2Tyr in the studied extracts.

Figure 1

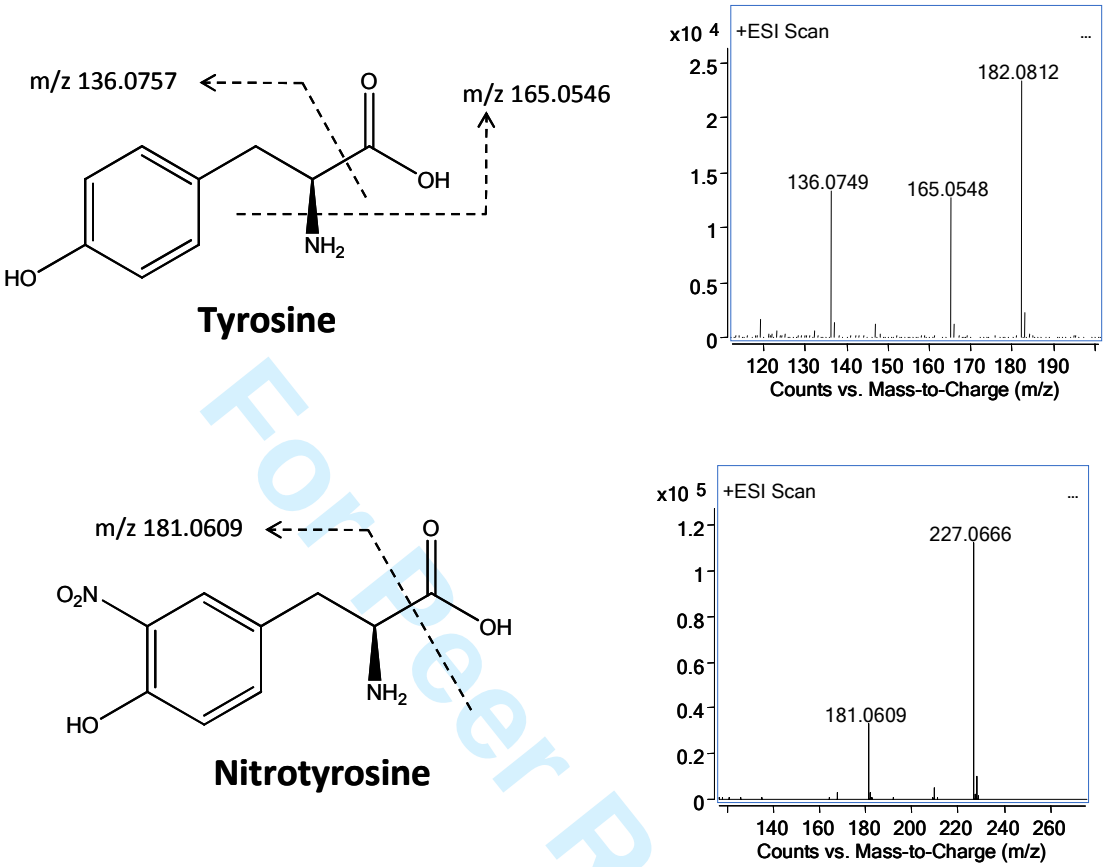


Figure 2

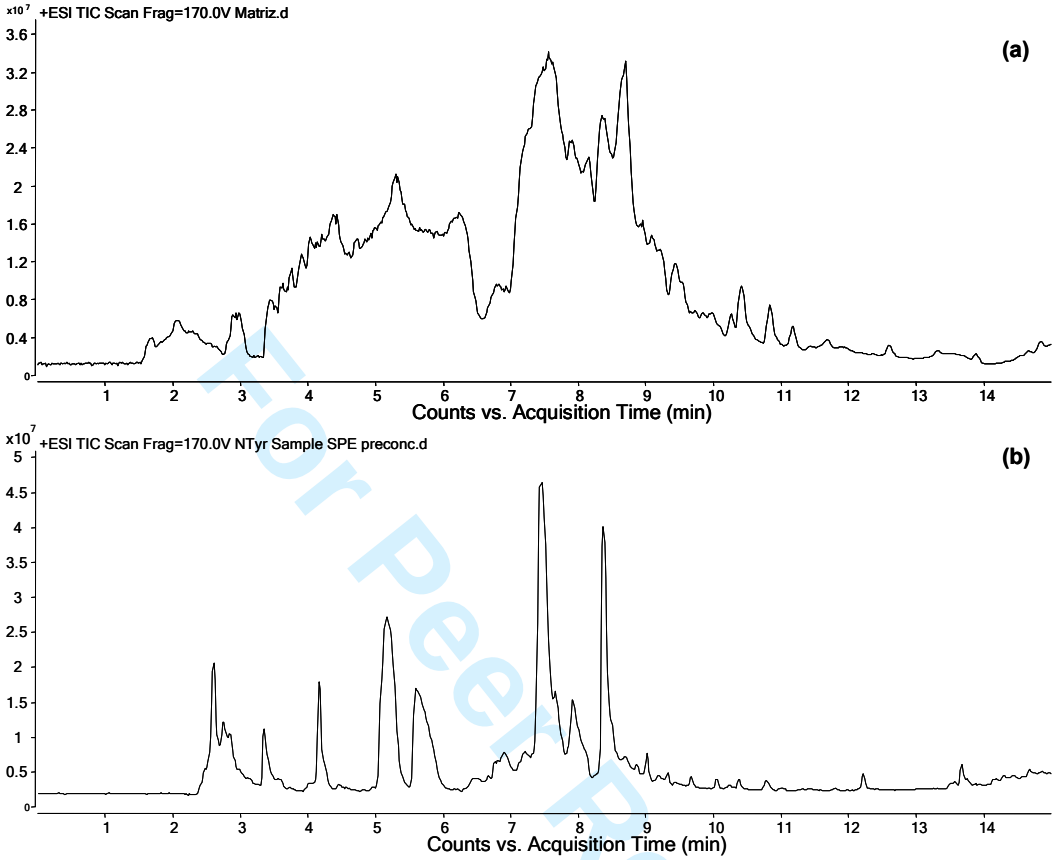


Figure 3

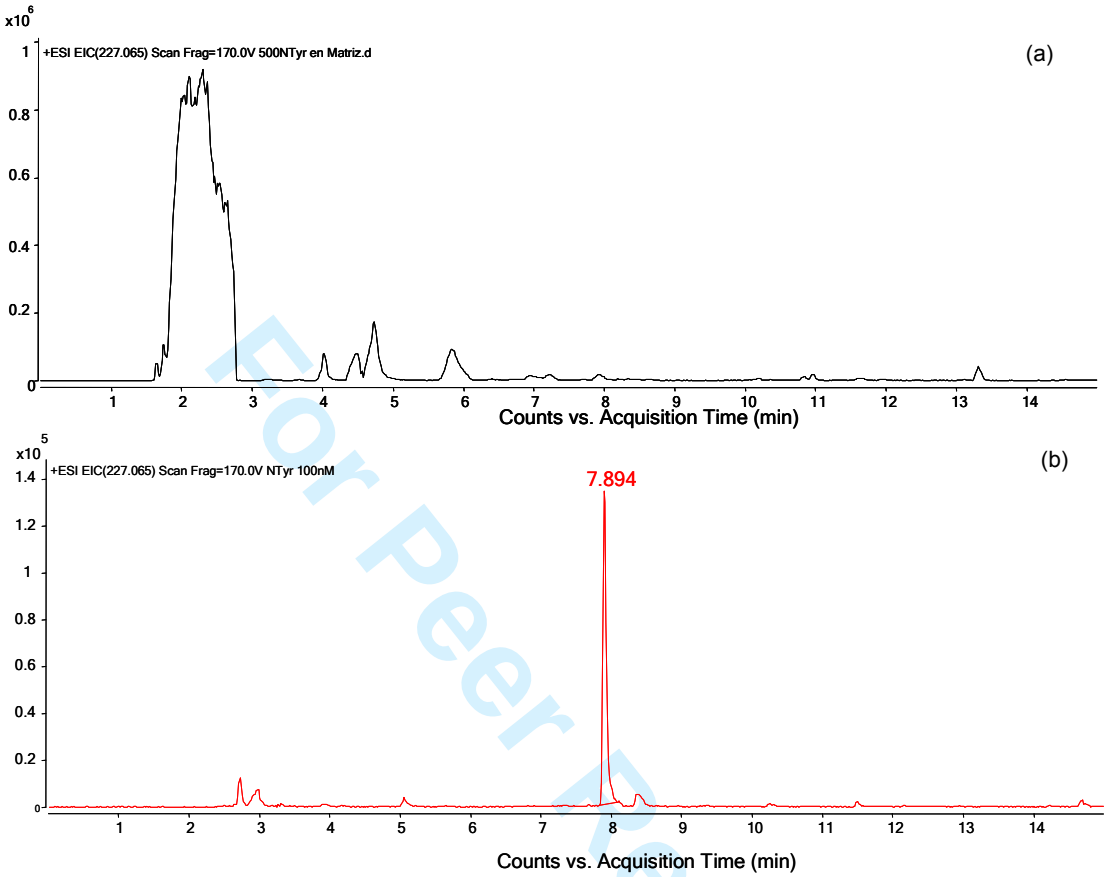
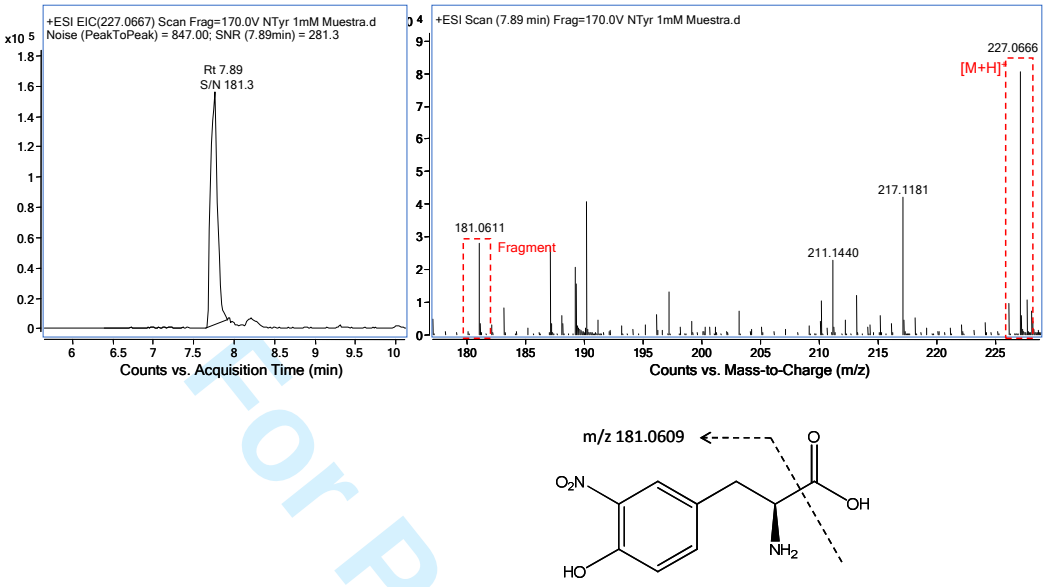


Figure 4



UNIVERSIDAD DE JAÉN
Facultad de Ciencias Experimentales
Dpto. de Quimica Fisica y Analitica

To: Prof. Aldo Roda
Analytical and Bioanalytical Chemistry
Editorial Office

From: Prof. Antonio Molina Díaz
University of Jaén
Department of Physical and Analytical Chemistry
E-23071 Jaén (SPAIN)

Jaén, Spain, 12th June, 2012

Dear Prof. Roda,

This letter accompanies the online submission to *Analytical and Bioanalytical Chemistry* of revised version of the manuscript **ABC-2012-00704** entitled **"Determination of nitrotyrosine in *Arabidopsis thaliana* cell cultures with a mixed-mode solid-phase extraction clean-up followed by liquid chromatography time-of-flight mass spectrometry"**, by P. Berton *et al.*

In this version we have carefully taken into account all the comments made by the reviewers. Enclosed are responses to the reviewers' comments.

We hope this revised version of the manuscript will now be accepted for publication in *Analytical and Bioanalytical Chemistry.*

Looking forward to hearing from you,

Sincerely yours,

Prof. Antonio Molina Díaz

MANUSCRIPT ABC 00704-2012-R1

RESPONSE TO REVIEWER A' COMMENTS

We appreciate the reviewer's comments and suggestions. Following are our responses.

Query. ***Overall the manuscript lacks novelty and does not significantly contribute to the knowledge of the existing literature. Due to its biological significance, the determination of nitrotyrosine in biological samples has been extensively reported in the literature, including the GC-MS and LC-MS approaches with the cleanup or enrichment methods. In this manuscript, the authors implemented the mixed-mode SPE for sample cleanup before LC-TOF-MS analysis, which didn't add any novel aspect to the available method. Also, the authors did not provide detailed comparison between the method they developed and those in the literature to demonstrate the performance.***

Response. We have performed a dedicated literature search and there are not that many papers especially dealing with LC-Electrospray-MS for nitrotyrosine (NTyr) detection. Actually, to our knowledge, there is no article dealing with LC-MS determination of NTyr in any plant material. We only found methods for biological samples such as urine or mice liver. Therefore, the present article is quite difficult to compare with existing literature because there is nothing similar published before. With regards to matrix effects and sample preparation considerations we also have not found any discussion throughout the literature.

Arabidopsis Thaliana is a model sample used in many studies (*ca.* 2000 references per year according to Scopus database). Therefore, the development of methodologies able to handle the determination of metabolites and other small molecules of interest in *Arabidopsis Thaliana* by means of LC-MS is a relevant topic that may attract readers and citations. We consider it could be a positive contribution to *Analytical and Bionalytical Chemistry*, given the scarce literature available up to date.

RESPONSE TO REVIEWER B' COMMENTS

We appreciate the reviewer's comments and suggestions. Following are our responses.

-Query 1 (Q1) Abstract (page 2) and page 13: The statement "A linear relationship between concentration of peroxide and NO_2Tyr/Tyr ratio was observed" Was not clear to me as only 2 peroxynitrite conditions were used. Could the authors clarify?

Response 1 (R1). We agree with the referee. A dedicated study with more data would be required. This sentence has been deleted from the abstract in the revised version of the manuscript. The sentence from page 13 has been rewritten as suggested by the reviewer.

Q2. Page 5: Could the authors justify that they compared the extraction efficiency obtained with columns with different amounts of solid phase?

R2. Two different amounts were used simply because these are the available formats from the different suppliers/manufacturers. The sample volume loaded is relatively low whatsoever. Therefore, the amount of material is not a limiting factor to the extent that using 150 mg or 200 mg may be eventually critical. On the other hand, the SPE cartridge with 30 mg of sorbent was associated with 2.5-mL sample extract loaded, while the cartridges tested with 150-200 mg of sorbent were evaluated with 10-mL sample extract loaded. We do not consider this could be a critical issue during the study.

Q3- ***Page 7: Why did the authors describe only one of the extraction procedures? It may be worthwhile to describe the other extraction procedures even as supplementary information.***

R3. We agree with the referee. A brief description of the additional procedures tested but not selected as the optimum have been included in section 2.3 in the revised version of the manuscript as suggested by the reviewer.

Q4. Page 8: Was the negative ion mode finally used in the method?

R4. This is a typo. We have addressed this in the revised version. Only positive ion mode detection was used in the final method

Q5. Page 9: Could the authors provide proportions between precursor-ions and their in-source fragments in Table 1 and fully clarify which ions are used for quantitation for both NO_2Tyr and Tyr? Could $[M+H]^+$ be used for both confirmation and quantitation (it appeared to me that confirmation should have been done with one fragment)?

R5. We have included the requested information of relative abundance of protonated molecules and fragments ions in Table 1 in the revised version of the manuscript. The more abundant ion (protonated molecule) is always used as quantitation ion and the accurate mass measurement is used along with retention time for identification purposes, while the fragment ion accurate mass measurements are used for further confirmation. This comment has been also included in the revised version of the manuscript (Section 3.1)

Q6.- Page 11, line 1: Was the pH optimization performed also with other SPE material or only with Strata?

R6. The study of pH was performed with both cartridges studied. This has been included in the revised version of the manuscript as suggested by the reviewer.

Q7.- What would the authors tell/conclude from the TIC current is similar to the raw extract despite the preconcentration factor (5:1)?

R7. The Total Ion Current chromatogram accounts for the sum of the signals for all coeluting ions at each individual acquired spectrum. This can be considered an indicator of the complexity of a matrix. Higher matrix contents (eg. Ratio g sample/mL extract) will lead to higher TIC currents.

Therefore, if you intentionally preconcentrate a sample (eg. 5-fold), an increase of the TIC current should be expected. If the TIC signal is reduced or is in the same signal range of the original unpreconcentrated sample, this indicates that a convenient loss of matrix constituents has been achieved during this preconcentration stage. This was exactly the case of the present study and it is clearly illustrated in Figure 2. A comment clarifying this aspect has been included in the revised version of the manuscript.

Q8.- Page 12, line 4: Could the authors clarify/explain in more detail their strategy and the concentration ranges used for the calibration curves (especially because of a higher Tyr concentration over NO_2Tyr in real-life samples)?
Q11.- Table 2: Could the authors clarify that a 200:1 dilution was applied for quantitation purposes?? Did the authors envisage using heavy stable-isotope (i.e., C13 C9 tyrosine)?

R8-R11. With the TOF instrument used with 2.5-3 orders of linear dynamic range (and probably with most mass spectrometers) it is very difficult to accurately measure both nitrotyrosine and tyrosine in the same run, because there are *ca.* 3-orders of magnitude between both concentration levels. Therefore, unless both Tyr/NTyr concentration levels in the sample are known *a priori*, it is difficult to set the conditions and sample preparation/preconcentration level required to allow the NTyr/Tyr determination in a single run. Perhaps, by using a triple quadrupole instrument, best suited to handle such concentration levels differences, this issue may eventually be circumvented.
For this reason, and also to skip matrix effects for accurate tyrosine quantitation, a 1:100 dilution of the extract was also analyzed which enabled the determination of tyrosine without matrix effects (external calibration was found appropriate). Given the fact, that this nitrosation experiments are not routine and may not require too many analyses, this additional injection does not represent a big disadvantage considering that standard addition method must be applied for NTyr determination. For a dedicated routine work, the use of triple quadrupole (as usual) would be required to skip this issue.

With regards to the use of deuterated internal standards, the main drawback is prize and availability. The use of NTyr-d_9 has been previously reported [Ishii et al., 2006] for nitrotyrosine detection by LC-MS. On the other hand, from our experience when dealing with such comp matrices with varying analyte concentration levels, these labeled standard not necessarily provide that accurate results and sometimes the use of standard addition method is required anyway. We have observed poor quantitative performance under similar conditions with labeled atrazine and imazalil, when dealing with relatively high matrix effect (signal suppression) as it is the case. For this reason and also because of the high prize we did not test these isotopically labeled standards.

In the revised version of the manuscript (section 3.3), we have included a sentence with the explanation of the different concentration levels and the need of injecting diluted extracts for tyrosine detection.

Q9. - Page 12, line 20: What were the low concentration levels and the most abundant ions used (please provide more details)? As well, the authors should provide references for the previously reported method?.

R9. We mistakenly omitted in this section (page 12, line 20) a reference **[Ishii et al., J. Pharm Biomed. Anal. 41 (2006) 1325]** that is somewhat related with the present study. It is the only reference devoted to LC-MS determination of nitrotyrosine. This is to our knowledge the more relevant article (in terms of analytical performance) dealing with LC-MS(MS) method development for nitrotyrosine detection, although the matrix studied is not related at all with that from the current manuscript. This reference has been cited in the revised version of the

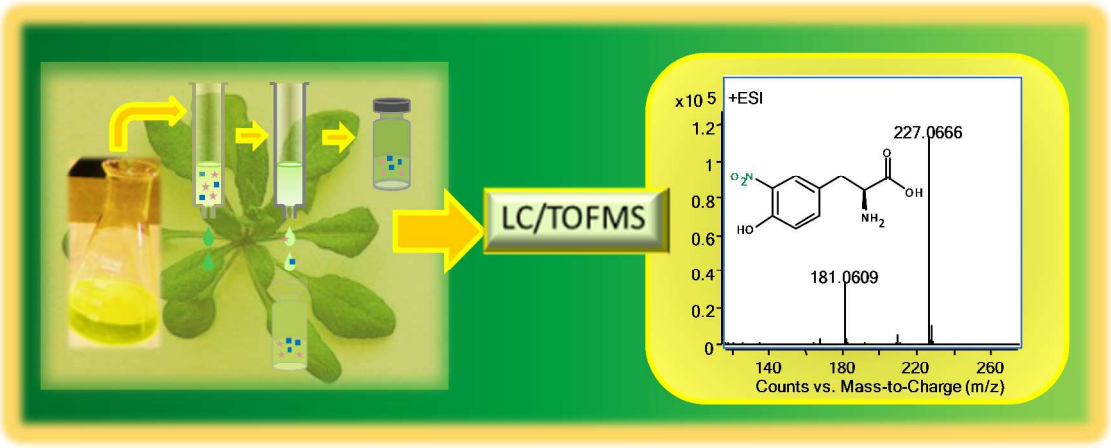
manuscript and the performance of the proposed method critically discussed and evaluated in Results and discussion (section 3.3).

Q10- Page 13, lines 4-18: This paragraph was not clear to me. Were the hypocotyl samples described before? Figure 4 was not described/indicated in the text?
R10. This is a typo revised in the final version of the manuscript ("hypocotyl samples" has been deleted).

ADDITIONAL COMMENTS
Typos:
- Page 3, line 16: the first sentence of the paragraph should be rephrased for better understanding.
- Page 3, line 24: ?data indicates?...
- Page 4, line 7: please clarify ?its?
- Page 4, line 23: ?GC-based methods?
- Page 6, line 15: ?[25].?
- Page 7, line 2: "strong cation-exchange"..."reversed-phase"
- Page 10, line 4, line 9, line 13, line 15, line 20: "Figure 1"..."cation-exchangers"... "reversed-phase"... "hydrophilic and lipophilic"..."Oasis MCX"

Response. We have revised the manuscript as suggested by the reviewer and all the typos marked have been addressed in the revised manuscript.

Graphical abstract



Determination of nitrotyrosine in *Arabidopsis thaliana* cell cultures with a mixed-mode solid-phase extraction cleanup followed by liquid chromatography time-of-flight mass spectrometry

Paula Berton[a,b,c], Juan C. Domínguez-Romero[a], Rodolfo G. Wuilloud[b,c], Beatriz Sánchez-Calvo[d], Mounira Chaki[d], Alfonso Carreras[d], Raquel Valderrama[d], Juan C. Begara-Morales[d], Francisco J. Corpas[d], Juan B. Barroso[d], Bienvenida Gilbert-López[a], Juan F. García-Reyes[a] and Antonio Molina-Díaz[a,*]

[a]*Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, Campus Las Lagunillas, Edif. B-3, 23071 Jaén, Spain.*
[b]*Analytical Chemistry Research and Development Group (QUIANID), Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Padre Jorge Contreras 1300, Parque Gral. San Martín, C.P. M5502JMA Mendoza, Argentina.*
[c]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.*
[d]*Grupo de Señalización Molecular y Sistemas Antioxidantes en Plantas, Unidad asociada al CSIC (EEZ), Departamento de Bioquímica y Biología Molecular, Universidad de Jaén, Spain.*

Keywords: nitrotyrosine, *Arabidopsis thaliana*, nitrosative stress, liquid chromatography, mass spectrometry, solid-phase extraction.

*Corresponding author: Prof. Antonio Molina-Díaz. Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, 23071 Jaén, Spain. Tel.: (+34) 953 212147; Fax: (+34) 953 212940. E-mail:amolina@ujaen.es

Abstract

In this work, a method for the determination of trace nitrotyrosine (NO_2Tyr) and tyrosine (Tyr) in *Arabidopsis thaliana* cell cultures is proposed. Due to the complexity of the resulting extracts after protein precipitation and enzymatic digestion and the strong electrospray signal suppression displayed in the detection of both Tyr and NO_2Tyr from raw *Arabidopsis thaliana* cell culture extracts, a straightforward sample cleanup step was proposed. It was based on the use of mixed-mode solid-phase extraction (SPE) using MCX-type cartridges (Strata™-X-C), prior to identification and quantitation using fast liquid chromatography electrospray time-of-flight mass spectrometry (LC-TOFMS). Unambiguous confirmation of both aminoacids was accomplished with accurate mass measurements (with errors lower than 2 ppm) of each protonated molecule along with a characteristic fragment ion for each species. Recovery studies were accomplished to evaluate the performance of the SPE sample preparation step obtaining average recoveries in the range 92–101%. Limit of quantitation (LOQ) obtained for NO_2Tyr in *Arabidopsis thaliana* extracts was 3 nmol L^{-1}. Finally, the proposed method was applied to evaluate stress conditions of the plant upon different concentrations of peroxynitrite, a protein-nitrating compound, which induces the nitration of Tyr at the nanomolar range. Detection and confirmation of the compounds demonstrated the usefulness of the proposed approach. ~~A linear relationship~~correlation ~~tendency between concentration of peroxynitrite and NO_2Tyr/Tyr ratio was observed.~~

Keywords: nitrotyrosine, *Arabidopsis thaliana*, nitrosative stress, liquid chromatography, mass spectrometry, solid-phase extraction.

1. Introduction

Tyrosine (Tyr) nitration is becoming increasingly recognized as a prevalent, functionally significant post-translational protein modification (PTM), which can occur in cells during oxidative stress and over-production of nitric oxide [1]. This modification is involved in the control of fundamental cellular processes including cell cycle, cell adhesion and cell survival, as well as cell proliferation and differentiation [2]. The addition of NO_2 group to the ortho-position of Tyr confers particular physicochemical properties to ~~the~~ the modified amino acid and the corresponding proteins, as a consequence of ~~a~~ pK_a ~~-~~ reduction ~~of the pK_a of its hydroxyl group of~~ about three units [3]. These changes in protein conformation may have important functional consequences, such as a loss, an increase, or no effect on protein function [4-7]. Elevated levels of 3-nitrotyrosine (NO_2Tyr) have been reported in a range of pathological conditions including inflammatory, neurodegenerative, and cardiovascular disorders, among others [8,9]~~[8, 9]~~. Moreover, emerging data indicate a novel biological function for Tyr nitration in the regulation of immune responses [1].

~~For these reasons and for its chemical stability, NO_2Tyr is considered not only the most important biomarker for identification and quantitation of cellular processes, associated to reactive nitrogen species (RNS) occurrence, that lead to PTM of proteins, but also Tyr nitration itself may impair cell function [10].~~ Therefore, in mammals Tyr nitration is being intensively studied because it can be used as a biomarker not only of nitrosative stress, but also of certain ~~patholog~~pathological and physiological conditions~~ies and nitrosative stress~~ [10,11]~~[11]~~. Additionally, new studies emphasize the possible involvement of Tyr nitration in signaling pathways mediated by NO [1].

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On the other hand, in plants the information available on protein nitration under normal conditions is rather limited [12]. Even though previous data indicate the existence of a basal nitration present in the plant tissues analyzed, there are published data which indicate that an increase in the number of proteins or an intensification of specific proteins resulting from Tyr nitration could be considered as an indicator of nitrosative stress in plants [13,7,14,15]~~[7, 13-15]~~. Therefore, protein Tyr nitration might be a good starting point in the search of nitrosative stress markers in plant cells [13]. Nevertheless, since the actual number of nitrated Tyr residues in proteins is unknown, it is by far more preferable to use molar ratio of nitrated Tyr residues to non-nitrated Tyr residues [16]. However, the overall concentration of nitrated Tyr residues is typically low ~~However, its overall yield is typically low~~ [9]. Hence, assays applied to the analysis of NO_2Tyr in biological samples must offer a low limit of detection, accuracy and precision.

Detection of NO_2Tyr in biological samples has been extensively reported in the literature. These methods fall into two basic categories: molecular analysis using NO_2Tyr antibody-staining techniques [13] and chemical analysis using HPLC and GC [14], mainly using mass spectrometers as detectors. The source and nature of analytical problems, shortcomings and pitfalls associated with NO_2Tyr determination have been reviewed by Duncan [17] and Tsikas [16,18]~~[16, 18]~~. The main drawbacks are both the low abundance of nitrated species and lack of efficient enrichment methods [2].

Mass spectrometry (MS) is a powerful analytical technique with inherent selectivity, sensitivity and precision when applied to NO_2Tyr determination. Moreover, NO_2Tyr immunoassays, unlike GC-MS and LC-MS based-methods, cannot provide important information about NO_2Tyr/Tyr ratio [17,19]~~[17, 19]~~. In view of the complexity inherent in the determination of NO_2Tyr, and the confounding results

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evident in the literature, MS has thus been adopted by several groups [18]. Furthermore, comparing with ~~GC-~~GC-based- methods, LC-MS methods offer advantages such as that it is no longer necessary to modify the analyte to impart volatility. Because chemical manipulation can be eliminated, sample handling, the potential for side-reactions, losses and contamination are also minimized [17]. These complex matrices require, however, a careful consideration in order to evaluate and eliminate matrix effects when developing an LC-MS assay, particularly because of matrix effects/signal suppression, the Achilles' heel of quantitative LC–Electrospray(ESI)-MS [20]. In LC-ESI-MS, methods skipping sample cleanup stages lead to poor analytical performance, in particular, when complex matrices are addressed and sensitive methods are needed. In the present work, a sensitive, simple and specific sample preparation method based on mixed-mode solid-phase extraction (SPE) was developed for the accurate quantification of trace NO_2Tyr in plant tissues by LC-TOFMS using *Arabidopsis thaliana*, as model sample.

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2. Materials and methods

2.1. Reagents and Materials

Tyrosine (Aldrich) and 3-nitro-L-tyrosine (Aldrich) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the studied compounds (1.77 mmol L^{-1} of nitrotyrosine and 1.10 mmol L^{-1} of tyrosine) were prepared in water and stored at −20 ºC. HPLC-grade solvents acetonitrile (Chromasolv® Gradient) and methanol (Chromasolv® for HPLC) were purchased from Sigma-Aldrich. Formic acid was obtained from Fluka (Buchs, Switzerland). A solution of 5% (v/v) ammonium hydroxide (Sigma-Aldrich) in methanol was employed in SPE procedure. A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water. The SPE cartridges evaluated for comparing

cleanup were Strata™-X-C cartridges with a capacity of 30 mg, (Phenomenex, Torrance, CA, USA); AccuBOND^{II} SCX cartridges (200 mg, 3 mL) were acquired from Agilent Technologies (Waldbronn, Germany); Oasis® MCX SPE cartridges (150 mg, 6 mL) and Oasis® HLB (200 mg, 6 mL) were purchased from Waters (Milford, MA, USA). Additionally, a Supelco (Bellefonte, PA, USA) Visiprep™ SPE vacuum system was also employed.

2.2. *Sample preparation and treatment*

Arabidopsis thaliana L. (Columbia ecotype) cell suspension culture was kindly provided by the Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Salamanca (Spain). The culture was maintained in 200 mL of liquid growth medium [21,22]~~[21, 22]~~ by gentle agitation at 120 rpm and 24 ºC under continuous illumination (50 μE m^{-2} s^{-1}) in an incubator shaker. Cells were sub-cultured with a one-twentieth dilution every seven days. The treatment of the cell culture was performed as described by Chaki *et al.* [23,24]~~[23, 24]~~. The cell culture was treated with different concentrations of peroxynitrite by infusion for one hour in the same cell culture conditions. After an hour, cell suspension culture was grounded and homogenized in liquid nitrogen using a mortar and pestle. The resulting powder was suspended into 1/2 (w/v) digestion buffer (50 mmol L^{-1} sodium acetate, pH 6.5) according to Hensley *et al.* [25]. Homogenates were then filtered through one layer of Miracloth (Calbiochem, San Diego, CA, USA) and centrifuged at 3000 g for 10 min. The supernatant proteins were then precipitated by the addition of 10% trichloroacetic acid (TCA). After incubation at 4 ºC for 20 min, the samples were centrifuged at 14,000 g for 10 min. Protein pellets were washed twice with acetone at –20 ºC, air-dried, and re-suspended in 1 mL of digestion buffer containing 4 mg of pronase (Calbiochem), and incubated at 50 ºC for 30 h with gentle stirring. The digested samples were treated with

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10% TCA at 4 ºC for 20 min followed by centrifugation at 14000 g for 10 min. The pH of the supernatant was adjusted to 3. The supernatants were passed through 0.45 µm PVDF filter.

2.3. Mixed-mode solid-phase extraction cleanup

Strata™-X-C cartridges cation-exchange cartridges with a capacity of 30 mg, with a mixed-mode stationary phase (strong ~~cation~~ cation-exchange and ~~reverse~~ reverse-phase) were used to perform the SPE-based cleanup. The cartridges were placed on a vacuum SPE manifold being preconditioned with 1 mL of methanol and 1 mL of 0.1 N HCl in water at a flow rate of 2 mL min^{-1}. Subsequently, 2.5 mL of plant extract (previous adjustment to pH 3) was loaded onto the SPE cartridge, at a flow rate of 1 mL min^{-1}. Finally, the sample was eluted into the test tube using twice 2 mL of 5% (v/v) ammonium hydroxide in methanol at 1 mL min^{-1}. The eluate pH was then neutralized by vacuum evaporation of the ammonium hydroxide. Samples were evaporated until near dryness by a gentle nitrogen stream and reconstituted with 500 µL of methanol:H_20 (20%, v/v) (final preconcentration factor 5:1) prior to analysis. The extract was finally filtered through a 0.45 µm PTFE filter (Millex FG, Millipore, Millford, MA, USA). For validation and quantitation purposes, matrix-matched standards were prepared by spiking the extracts with appropriate volume of NO_2Tyr working standard solution before the SPE extraction procedure.

Additional experiments were also performed using cation-exchange and reverse-phase type SPE cartridges). Two cation-exchange cartridges (AccuBOND^{II} SCX (200 mg, 3 mL) and Oasis MCX SPE cartridges (150 mg, 6 mL)) were also tested although they were not selected as the final optimized method. The cation-exchange SPE cartridges were washed with MeOH (5 ml) and 5 mL of 0.1 M HCl in water at a flow rate of 2 mL

min^{-1}. Subsequently, 10 mL of plant extract (previous adjustment to pH 3) was loaded onto the SPE cartridge, at a flow rate of 1 mL min^{-1}. Finally, the sample was eluted into the test tube using twice 2.5 mL of 5% (v/v) ammonium hydroxide in methanol at 1 mL min^{-1}. The resulting extract were evaporated until near dryness by a gentle nitrogen stream and reconstituted with 2 mL of methanol:H_20 (20%, v/v) prior to LC-MS analysis.

Besides, a hydrophilic-lipophilic balanced Oasis HLB cartridge was also tested (200 mg, 6 mL). The cartridge was washed with MeOH (5 ml) and 5 mL of mQ water at a flow rate of 2 mL min^{-1}. Subsequently, 10 mL of plant extract (previous adjustment to pH 3) was loaded onto the SPE cartridge, at a flow rate of 1 mL min^{-1}. Finally, the sample was eluted into the test tube using twice 5 mL methanol at 1 mL min^{-1}. The resulting extract was evaporated until near dryness by a gentle nitrogen stream and reconstituted with 2 mL of methanol:H_20 (20%, v/v) prior to analysis.

2.4. Liquid chromatography electrospray time-of-flight mass spectrometry

The separation of the species from the whole SPE extracts was carried out using an HPLC system (consisting of vacuum degasser, auto-sampler and a binary pump) (Agilent 1290 Infinity, Agilent Technologies, Santa Clara, CA, USA). Optimization studies were carried out with standard mixtures performing chromatographic separation on an Agilent ZORBAX Eclipse XDB-C_{18}, Rapid Resolution HT (4.6 × 100 mm, 1.8 µm). For the elution, 0.1% (v/v) formic acid in high purity water (mobile phase A) and acetonitrile (mobile phase B) were used as solvents at a flow rate of 500 µL min^{-1}. The gradient program started at 5% B and after 2 min of isocratic run solvent B was

increased linearly and reached 50% at 10 min, then 100% at 13 min. Finally, 100% B was kept constant for two minutes (until 15 min) and after the acquisition 10 min post time was set for the equilibration of the initial solvent composition. The column temperature was maintained at 24 ºC and an injection volume of 20 µL was used in all experiments.

The HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in positive or negative ion mode, using the following operation parameters: capillary voltage, ±4000 V; nebulizer pressure, 40 psig; drying gas flow rate, 9 L $min^{-1;}$ gas temperature, 325 ºC; skimmer voltage, 65 V; fragmentor voltage (in-source CID fragmentation), 170 V in positive ion mode. LC−MS accurate mass spectra were recorded across the range of 50−1000 m/z. Accurate mass measurements of each peak from the total ion chromatograms (TICs) were obtained using an automated calibrant delivery system to provide the correction of the masses. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low flow (approximately 10 µL min^{-1}) of a calibrating solution which contains the internal reference masses purine ($C_5H_4N_4$ at m/z 121.050873) and HP-0921 ([hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at m/z 922.009798)). The full-scan data recorded was processed with Agilent Mass Hunter software (version B.04.00). Extracted ion chromatograms were obtained throughout the study using ±5 mDa mass window.

3. Results and discussion

3.1. *Identification and confirmation of tyrosine and nitrotyrosine by LC–ESI-TOFMS: in-source CID fragmentation and accurate mass measurements*

The fragmentor voltage is the parameter that establishes the extent in which in-source CID fragmentation is carried out, which may have a strong influence on the sensitivity and relative abundance of protonated molecules [26]. Due to the low masses of both Tyr and NO_2Tyr, the fragmentor voltage was set at 170 V (mild conditions), as a compromise value between sensitivity for quantitation and additional mass spectrum information for confirmation purposes. Using the selected conditions, useful fragmentation was obtained. Table 1 shows the fragmentation of Tyr and NO_2Tyr and the relative abundances of the different species formed.

Primary identification of both compounds was performed basically by retention time matching and accurate mass measurements of the targeted protonated molecules and their main fragment ions. By using high resolution mass spectrometry data with high mass accuracies, as those shown in Table 1, unambiguous identification of the targeted species was accomplished. For identification and quantitation purposes, extracted ion chromatograms (ECIs) were employed, using a mass-window width of 5 mDa ($[M+H]^+$ ± 5 mDa). The protonated molecule ($[M+H]^+$) was used for both ~~confirmation~~ identification and quantitation purposes for NO_2Tyr and Tyr. Accurate-mass data from additional fragment ions available for NO_2Tyr and Tyr were used for further confirmation. Figure 1 show~~s~~ LC-TOFMS mass spectra of Tyr and NO_2Tyr obtained in the positive ionization mode.

<Figure 1 and Table 1>

3.2. *Sample treatment and recovery studies*

After unsuccessful attempt of direct injection of the *Arabidopsis thaliana* extract (Figures 2 and 3), in order to eliminate additional interfering species from the sample

extract, a SPE cleanup step was evaluated and included in the method. Although slightly more time-consuming, the improvement in chromatographic performance provided by the SPE step was significant. Additionally, the extraction method could be easily automated using a SPE–LC–TOFMS assembly, thus increasing the throughput and automation degree of the procedure. Inspection of the structure, solubility data, and acid/base properties of Tyr and NO_2Tyr suggests that it can be extracted by different mechanisms. For example, ionic interactions could be increased through pH variation. Furthermore, the aromatic side chain of Tyr and NO_2Tyr (Fig. 1) can be involved in stacking (non-polar) interactions with other aromatic side-chains; and the reactive hydroxyl group can be involved in polar interactions such as hydrogen bonding. Therefore, different sorbent materials with non-polar, polar, or ~~ion~~ ion-exchange properties were evaluated.

Among strong ~~cation~~ cation-exchangers (SCX), a cartridge based on silica (AccuBOND^{II} SCX) was tested. It is generally employed to extract positively charged basic compounds. Moreover, this benzene-sulphonic acid-based sorbent has significant non-polar secondary interactions. Different cartridges with a mixed-mode stationary phase (MCX) with reverse-phase and cation-exchange dual functionality, such as Oasis® MCX and Strata™-X-C were also evaluated. Besides, HLB cartridges, which have both hydrophilic and lipophilic~~hydrophobic~~ properties, generally employed to extract a variety of polar and non-polar compounds, were also considered. The cleanest chromatograms were obtained when cation exchange-based materials were employed. Among ~~cation exchange based~~these materials, best recoveries were obtained when Strata™-X-C cartridges were employed (91 and 83% recovery for Tyr and NO_2Tyr for Strata versus 61 and 46% for Tyr and NO_2Tyr with Oasis MCX), and the extracts obtained were particularly clean. Therefore, in order to maximize the retentive

differences between the analytes and the vegetable matrix, Strata™-X-C cartridges were employed for isolate the analytes from the matrix. For the SPE step, 2.5 mL of vegetable matrix sample were selected as the loaded volume. The preconcentration factor achieved in the final extract (500 µL) was 5:1.

The pH is a significant variable when developing a SPE method. Interactions between the matrix components and the target analytes in biological samples may be disrupted by a change in pH [27]. Thus, spiked matrix stabilized at neutral (pH 7) and acidic (pH 3) pHs were evaluated for both MCX cartridges. A significant improvement on analytes recoveries was observed when Strata cartridges at acidic pH (85-90% recovery for Tyr and NO_2Tyr) were employed, comparing with those at neutral pH (lower than 10% for both analytes). –Therefore, pH from samples was adjusted to 3 before SPE.

Figure 2 shows a comparison of Total Ion Current Chromatograms (TICs) obtained from the raw *Arabidopsis thaliana* extract without further treatment (Figure 2a) and with the proposed SPE-based cleanup method (Figure 2b). The TICs accounts for the sum of signals for all coeluting ions at each individual acquired spectrum. This can be used as an indicator of the complexity of a matrix and to evaluate the degree of efficiency of a cleanup step. Note that the TIC obtained with the SPE procedure is cleaner (lower average signal) than the raw extract, even considering the 5:1 preconcentration factor, as shown in the chromatogram where major matrix peaks are baseline-separated and the TIC current is similar to the raw extract despite the preconcentration factor (5:1). With this SPE approach, the chromatogram region where NO_2Tyr is detected is free of several coeluting interfering species. In Figure 3, EICs for the detection of NO_2Tyr in the studied plant extracts obtained from (a) the raw extract without further treatment (500 nmol L^{-1} NO_2Tyr) and (b) with the proposed SPE-based

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cleanup method (100 nmol L^{-1} NO_2Tyr in the original *Arabidopsis thaliana* extract), are shown. It can be seen that at the studied concentration level (500 nmol L^{-1}), NO_2Tyr could not be detected in the raw extract due to strong signal suppression due to matrix coeluting components. In contrast, the identification of NO_2Tyr with the SPE approach was straightforward.

<Figure 2 and 3>

Even though after the sample treatment protocol a cleaner extract is obtained, the impact of the matrix on the ionization suppression/enhancement on the analytes was still significant. Therefore, a calibration with matrix-matched standards was employed throughout the study to minimize errors due to matrix effects.

<Table 2>

3.3. Analytical performance: in vitro nitration of Arabidopsis Thaliana cells

To evaluate the analytical features of the proposed method, calibration curves were constructed at different concentrations, in the range 10-500 and 50-2500 nmol L^{-1} of Tyr and NO_2Tyr respectively, using vegetable extracts to prepare matrix-matched standards at several concentration levels (2-100 and 10-500 nmol L^{-1} of Tyr and NO_2Tyr respectively), considering the SPE preconcentration factor. The results obtained are shown in Table 2 where the calibration curves are summarized together with the limits of quantitation (LOQs), matrix effects and relative standard deviation (RSD, %). The linearity of the analytical response across the studied range was excellent, taking into account that the calibration curves of the analyzed compounds showed correlation coefficients higher than 0.996. The RSD (n = 6) values for run-to-run study were 2.7 and 3.4% for Tyr and NO_2Tyr respectively. These results demonstrate the precision of the developed method and the potential of the proposed approach for quantitative

purposes. The LOQs were estimated as the minimum concentration of analyte corresponding to a signal-to-noise ratio (S/N) = 10:1. This was experimentally calculated from the injection of matrix-matched standard solutions at low concentration levels, using the more abundant ion for each compound based on the signal from high-resolution EICs with narrow mass windows (targeted mass ± 5 mDa). The LOQ obtained for NO_2Tyr was 3 nmol L^{-1}. Compared to the concentration levels that were achieved in previous reported methods for other biological matrices and considering the complexity of the studied extract, the LOQs reported here can be considered very satisfactory for the targeted application [28]. In the case of Tyr, LOQ could not be calculated because it is already present at large excess compared to NO_2Tyr in the studied samples. The LOQ of neat Tyr solvent standard was 10 nmol L^{-1} (without preconcentration step).

To evaluate the effectiveness of the extraction method, a recovery study was carried out. *Arabidopsis thaliana L.* cell culture ~~Hypocotyl samples~~ ~~were~~as incubated with two different concentrations of pure peroxynitrite (1 and 5 mmol L^{-1}), which had been shown to mediate Tyr nitration [24]. After sample preparation (explained on section 2.2), ~~Plant~~ the ~~extracts~~ aliquots were ~~then~~ spiked at different concentration levels (0.5 - 1 µmol L^{-1}) with the working standard solutions of Tyr and NO_2Tyr. The spiked samples were extracted with the SPE method described and then analyzed with the developed LC-TOFMS method. Due to the high concentration level differences between Tyr and NO_2Tyr (*ca.* 3 orders of magnitude) it was extremely difficult to accurately measure both Tyr and NO_2Tyr in the same run. This limitation is set by mass spectrometer, which usually features 2.5-3 orders of linear dynamic range. For this reason, and also to skip matrix effects for accurate Tyr quantitation, a 1:100 dilution of the extract was also analyzed which enabled the determination of Tyr without matrix

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effects, just by using external solvent-based calibration. A LC-ESI(+)TOFMS identification of NO_2Tyr in cell cultures exposed to peroxynitrite is shown in Fig. 4. The obtained recoveries rates for NO_2Tyr were in the range 92-101%, as shown in Table 3. These results show the feasibility of the studied extraction method for NO_2Tyr determination in the studied vegetable extracts. Besides, in the samples tested, both Tyr and NO_2Tyr were calculated for both experiments (1 and 5 mmol L^{-1} of peroxynitrite). Interestingly, a linear correlation tendency ~~linear relationship~~ between concentration of peroxynitrite and NO_2Tyr/Tyr ratio was observed. This proportional increase in the concentration of NO_2Tyr when increasing the concentrations of peroxynitrite corroborates the use of NO_2Tyr as a marker of nitrosative stress in plants.

<Figure 4 and Table 3>

4. Conclusions

The present work described a new method based on SPE and LC-TOFMS for quantitative analyses of NO_2Tyr and Tyr in *Arabidopsis thaliana* cell culture extracts. The method is simple, since it involves a quick cleanup step with SPE employing polymer-based cartridges before measurement with LC-TOFMS. Satisfactory recoveries were obtained for both studied compounds. Moreover, the high sensitivity obtained with the proposed method compares well with previous LC-MS/MS methods described for the analyses of NO_2Tyr and Tyr in biological matrices.

The potential of the proposed method was demonstrated by analyzing real samples with excellent selectivity and sensitivity, thus enabling the unambiguous identification, by means of accurate mass analysis, and quantitation of low levels of NO_2Tyr in *Arabidopsis thaliana* cell culture extracts.

The proposed LC-TOFMS method also offers the possibility of performing a posteriori (non-target) analysis of the samples, such as the search and identification of

others PTM-tyrosine compounds (such as sulfation, phosphorylation or carbonylation), involved in the regulation of a wide range of biological processes [29]. All the data are saved and can be re-examined to check for compounds that previously were not expected or were not subjected to control. This is an additional attractive feature that highlights the potential application of this method based on LC-TOFMS for studies related to PTM in biochemical laboratories worldwide.

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Table 1. Identification of tyrosine and nitrotyrosine by LC-TOFMS. Accurate mass measurements of the protonated molecules and the main fragment ions using *Arabidopsis thaliana* extracts[a]

Compound	t_R (min)	ion	Elemental compositions	Relative Abundance (%)	*m/z* theoretical	*m/z* experimental	Error	
							mDa	ppm
Tyrosine	3.43	$[M + H]^+$	$C_9H_{12}NO_3^+$	100	182.0817	182.0812	- 0.50	- 2.75
		$[M + H – NH_3]^+$	$C_9H_9O_3^+$	54	165.0546	165.0548	0.20	1.21
		$[M + H - HCOOH]^+$	$C_8H_{10}NO^+$	59	136.0757	136.0749	- 0.80	- 5.88
3-Nitrotyrosine[a]	7.99	$[M + H]^+$	$C_9H_{11}N_2O_5^+$	100	227.0662	227.0666	0.40	1.76
		$[M + H - HCOOH]^+$	$C_8H_9N_2O_3^+$	36	181.0608	181.0609	0.10	0.55

[a]Spiking level: 100 nmol L^{-1}.

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Table 2. Analytical parameters for the detection of nitrotyrosine in *Arabidopsis thaliana* cell culture extracts by LC–TOFMS.

Compound	**Conc. range tested** (nmol L^{-1})	**Regression equation**	**Matrix effect**[a] (Δ %)	**Linearity** (r)	**LOQ** (nmol L^{-1})	**RSD** (%)[c]
Tyrosine	10 - 500	$y = 2.624 \times 10^3 C + 7.97 \times 10^3$	Not calculated[b]	0.9997	Not calculated[b]	2.7
3-Nitrotyrosine	50 - 2500	$y = 1.36 \times 10^3 C + 3.21 \times 10^4$	0.07 (-93)	0.9972	3	3.4

[a]Ratio: matrix-matched calibration slope/solvent calibration slope.
[b]Matrix effect and limits of detection for Tyr could not be calculated because *Arabidopsis thaliana* cell culture extracts already contained Tyr between 100-250 µmol L^{-1}. A 200:1 dilution was applied for quantitation purposes. The LOQ of Tyr in solvent standard using the proposed method is 10 nmol L^{-1}.
[c]Concentration level: 100 nmol L^{-1}. n = 6

Table 3. Recovery studies on *Arabidopsis thaliana* cell culture extracts treated with peroxynitrite (1 and 5 mmol L^{-1}).

Sample treatment	Nitrotyrosine				Tyrosine (µmol L^{-1})	NO_2Tyr/Tyr ratio[a]
	Spiking level (µmol L^{-1})	Found (µmol L^{-1})[a]	Recovery (%)[a]	RSD (%)[a]		
Treatment 1:	0	0.196	---	---	189.33	0.001035
1 mmol L^{-1}	0.5	0.673	96.7	6.3		
peroxynitrite	1	1.100	92.0	7.8		
Treatment 2:	0	0.580	---	---	117.78	0.00492
5 mmol L^{-1}	0.5	1.090	101.0	3.1		
peroxynitrite	1	1.551	98.2	5.2		

[a] n=3

Figure captions

Figure 1. LC-Electrospray TOFMS mass spectra of Tyr and NO_2Tyr acquired in the positive ionization mode.

Figure 2. Total ion chromatograms (TICs) from the plant extracts obtained from the raw extract (a) without further treatment and (b) with the proposed SPE-based cleanup method.

Figure 3. Extracted ion chromatograms (EICs) for the detection of NO_2Tyr in the studied *Arabidopsis thaliana* cell culture extracts: (a) EIC obtained from the raw extract without further treatment ([NO_2Tyr] = 500 nmol L^{-1}); and (b) EIC obtained with the proposed SPE-based cleanup method ([NO_2Tyr] = 100 nmol L^{-1}).

Figure 4. LC-ESI(+)TOFMS identification of NO_2Tyr in *Arabidopsis Thaliana* cell cultures exposed to peroxynitrite (nitrating compound). Left: extracted ion chromatogram (m/z 227.0667); right: Electrospray TOFMS spectrum including ions at *m/z* 227.0666 and *m/z* 181.0611 that provides the unambiguous identification of NO_2Tyr in the studied extracts.

Figure 1

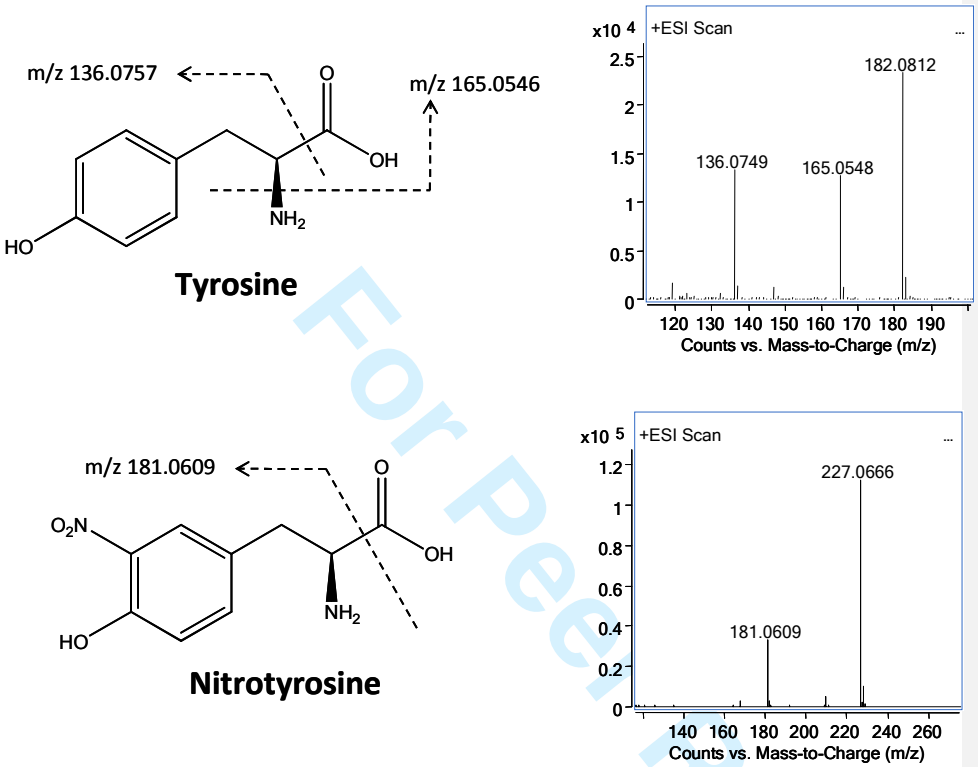

Figure 2

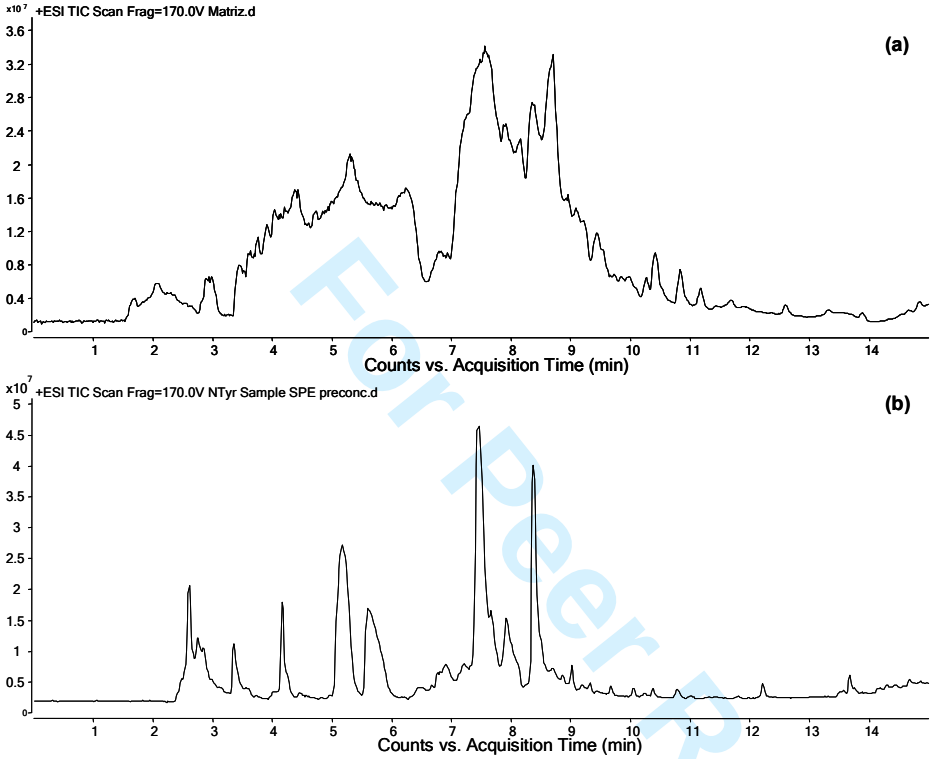

Figure 3

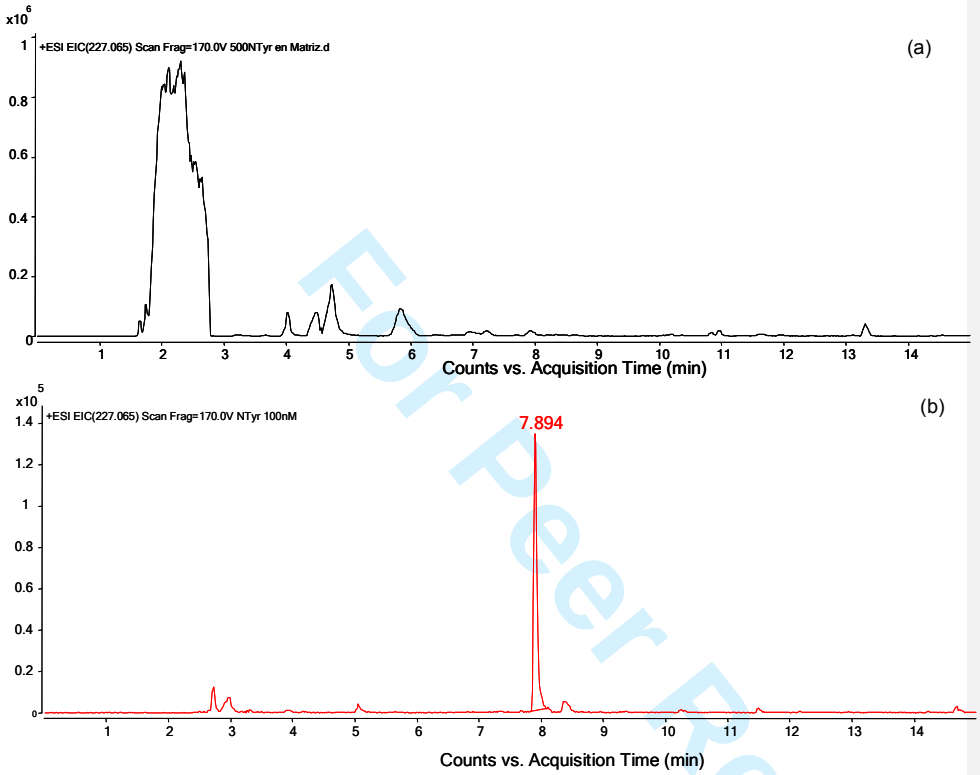

Figure 4